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Short Report

Exploring the limits for the survival of DNA in blood stains

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ABSTRACT

It is generally recognized that usable DNA may be retained in dry biological stains for years. We have explored the environmental limits for this property. Air-dried blood stains were incubated at different conditions of relative humidity (RH) and temperature. The quality of the extracted DNA was assessed by the ability to amplify 273 bp and 1600 bp DNA fragments by PCR, and by quantitative estimation of a 147 bp DNA fragment using real time PCR. Despite the fact that the availability of water is important for processes that degrade DNA, no significant difference was observed in the stability of DNA at 50%, 80% or 93% RH at room temperature or at 35 °C, and even the 1600 bp fragment was amplifiable after one year. Microbial growth was not observed at these conditions and the number of template molecules did not drop significantly over time. At 100% RH, however, microbial growth was observed after varying amounts of time. This may explain the decreased stability of DNA observed at these conditions. Even so, the 273 bp fragment was amplifiable for at least 3 months, and the 1600 bp fragment for at least two months. Microbial growth was not observed at higher temperatures (45–65 °C) at 100% RH, and the 1600 bp fragment was amplifiable after eight months at 45 °C, but only survived for one month at 55 °C or 65 °C. Thus DNA remains amplifiable in blood stains for many months, even at extreme RH and temperatures up to 45 °C. Even in humid climates the average RH is usually not more than 80% and RH rarely exceeds 93%; therefore we conclude that normal climatic conditions are not critical for the long time survival of DNA in untreated blood stains.

1. Introduction

The ability to extract and PCR amplify DNA from biological stains is a key element in Forensic Genetics. Fortunately DNA often survives for months or years at ambient conditions in untreated dry stains.[1–6] The effects of various parameters on the survival of DNA, such as UV-light, stain substrates, admixture of soil, age, humidity, and temperature have been previously assessed.[2,6–9] However, to the knowledge of the authors there is little information about the stability of DNA in stains when controlled conditions of humidity and temperature approach the extreme. Microbial enzymatic attack is of fundamental importance in the degradation of organic matter[10] but depends, among else, on the availability of water. Also many of the non-enzymatic processes that degrade DNA depend on the presence of water.[11,12] *A priori* one would therefore expect an accelerated rate of degradation of DNA in biological stains as the relative humidity approaches 100%. To determine the range of natural conditions that allow an adequate stability of DNA in stain material, blood stains without any stabilizing additives were incubated at various conditions of relative humidity (RH) and temperature. The quality and quantity of the remaining DNA was estimated by the ability to PCR amplify long and short fragments and by quantitative assessment using real time PCR.

2. Materials and methods

2.1. Chemicals

Sodium hydrogen sulphate, monohydrate ($NaHSO_4,H_2O$), ammonium sulphate ($(NH_4)_2SO_4$) and ammonium di-hydrogen phosphate ($NH_4H_2PO_4$) were of analytical grade and obtained from Merck KGaA, Darmstadt, Germany. All other chemicals were either of analytical grade or the highest purity available. Oligonucleotide primers and probe were obtained from TAGC, Copenhagen, Denmark.

2.2. Blood stains, constant RH, extraction of DNA

Human venous blood was drawn in dry BD Vacutainer® tubes (Becton & Dickinson, Franklin Lakes, NJ, USA) without additives and

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used immediately as follows. Five series of dry stains (I–V) were prepared by spotting five μl of blood onto pieces (4 × 4 mm) of Whatman filter paper No. 3 (Whatman International Ltd, Banbury, Oxon, UK) and air-dried in the open overnight. Series I–IV were similar in scope and initiated over the course of one year to assess the reproducibility of the results. Each of series I–IV was subdivided into stains incubated at either room temperature or 35 °C, and at each temperature stains were incubated at either 50%, 80% or 100% RH (series I and II), or at 80%, 93% or 100% RH (series III and IV) under non-sterile conditions. To increase the likelihood of contamination with spores and microorganisms, the stains of series III were exposed to the open air (mid June) in a nearby park (Fælledparken, Copenhagen) while the stains were still wet. Stains of series V were incubated at 100% RH and either 45 °C, 55 °C or 65 °C. Constant RH was maintained by storing the stains in sealed plastic boxes containing a beaker (Fig. S1) with either H_2O (100% RH) or saturated solutions of $NaHSO_4$,H_2O (50% RH), $(NH_4)_2SO_4$ (80% RH) or $NH_4H_2PO_4$ (93% RH).[13] At increasing amounts of time two stains were removed from each of the incubation boxes, and on the same day duplicate DNA extracts were prepared using the QIAamp® DNA Mini Kit including an overnight incubation of the stain with protease K at 56 °C (Qiagen GmbH, Germany). DNA was eluted from the silica-membrane-based columns with 200 μl Milli-Q water. All extracts were stored at −25 °C until use.

2.3. PCR

The ability to PCR amplify the DNA extracts was estimated for a 273 bp segment of exon 4 at the human HFE-locus, and a 1600 bp segment of the human ACP1 locus from intron 4 to exon 5 (Table S1). PCR was performed in 25 μl reactions using a Hybaid PCR Express thermocycler (Hybaid Ltd., Ashford, UK). The reactions contained 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatine, 0.2 mM of each dNTP, 0.4 μM of each primer and 1U of Taq DNA polymerase (Promega, Madison,WI). Thermocycling parameters were: 273 bp fragment, denaturation at 94 °C for 2 min, 33 cycles of 94 °C for 30 s, 67 °C for 30 s, 72 °C for 30 s; 1600 fragment, denaturation at 94 °C for 2 min, 33 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min. After the last cycle extension was continued at 72 °C for 7 min. The amount of amplicon was estimated on minigels, and band intensities compared to fixed amounts of a size marker (ΦX174 RF DNA *Hae*III-digested 72–1353 bp, New England Biolabs, Ipswich, MA).

2.4. Real time quantitative PCR analysis (qPCR)

qPCR was performed using the ABI-Prism-7000 SDS instrument and TaqMan Mastermix (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. qPCR reactions were set up in 25 μl volumes containing five μl of DNA extract. Primers and probe targeted a 147 bp segment in exon 4 of the human HFE-gene (Table S1). Standard dilutions of human DNA (100, 300, 1000, 3000, 10,000 copies/5 μl) were prepared using TaqMan control genomic DNA (10 ng/μl, Applied Biosystems, Foster City, CA) assuming that 1 ng DNA contains ∼300 haploid genomes or templates at any given autosomal locus (see below). All data points were determined in duplicate or triplicate.

2.5. Calculations

2.5.1. Estimation of the number of template molecules (T) in DNA

Under the assumption that the haploid human genome contains ∼3 billion base pairs,[14] the number of haploid genomes or template molecules (T) in 1 ng of DNA was calculated from $T = 1 \times N/3 \times 10^9 \times M \times 10^9$, where N is Avogadro's constant (6×10^{23}) and M is the average molecular mass of one base pair (650 g/mol). It will be seen that $T \sim 300$.

2.5.2. Estimation of the theoretical number of templates in extracts

The approximate quantity of DNA in each blood stain was estimated using the average white blood cell count, 7500 cells per μl of whole blood.[15] Thus, five μl blood used for each stain contain approximately $5 \times 2 \times 7500 = 75{,}000$ copies of any autosomal locus. Since each stain was extracted to 200 μl, the maximum amount of DNA template obtainable in five μl of extract (as used for qPCR) under optimal conditions was therefore estimated to $75{,}000 \times 5/200 = 2000$ copies. Since extraction efficiency is likely to vary between stains and qPCR measurements depend on multiple steps each introducing a variance it is stressed that quantitative results will only be a rough estimate of the actual amount of DNA present in the stain.

3. Results

To assess the stability of DNA in non-sterile blood stains, air-dried blood on filter paper was incubated at various conditions of controlled RH and temperature. The blood and the paper were without chelating agents or other stabilizing additives. The quality of the remaining DNA was monitored for one year by the ability to PCR amplify short and long DNA fragments.

In all four independent series (I–IV) DNA incubated at room temperature remained amplifiable for the entire experimental period as long as RH was less than 100% (93%, 80% and 50%). The same was the case at 35 °C for three of the four series (II–IV) while DNA in series I remained stable up to seven month (Figs. 1 and 2). At 100% RH the survival time was shorter, down to approximately four months for the 273 bp fragment, and approximately two months for the 1600 bp fragment, but large differences were observed among the series, and in one series (II) the short fragment remained amplifiable for the entire experimental period (Fig. 2). Microbial growth was observed at 100% RH after various amounts of time (1½–9 months), but in most cases growth only occurred on some of the filter paper pieces (Fig. 2 and Table S2). The occurrence of microbial growth was not more pronounced in series III, despite the fact that this series had been deliberately exposed to out-door conditions. Stains with microbial growth showed little or no surviving human DNA. Most important, growth was not observed on any of the stains incubated at 93% RH or less.

The stability of DNA at 100% RH was further tested at higher temperatures, 45–65 °C (series V, Fig. 3). At 45 °C the amplification of both fragments was still possible after eight to nine months. However, at 55 °C and 65 °C the stability dropped considerable, the 273 bp fragment was only detectable for three months, and amplification of the 1600 bp fragment was not possible after one month. Microbial growth was not observed at any of these elevated temperatures.

Real time PCR assessment of the quantity of intact DNA at selected time points was performed for series II–IV (Table S2). Although the number of extracted templates showed a considerable variation among the stains, the results generally paralleled the strength of the corresponding electrophoretic bands. The extracts of stains taken at time 0 showed the least variation among the series (range 329–443 copies/5 μl extract) and served as extraction controls. Since the optimal number of templates that was obtainable in five μl extract was ∼2000 copies (see Materials and methods), the extraction efficiency with fresh stains was only about 20%. Extraction of DNA using Qiagen silica-membrane-based spin columns may result in lower yields of DNA compared to other methods,[16,17] however, the QIAamp® DNA Mini Kit was chosen for the present work since it is a standardized and widely used

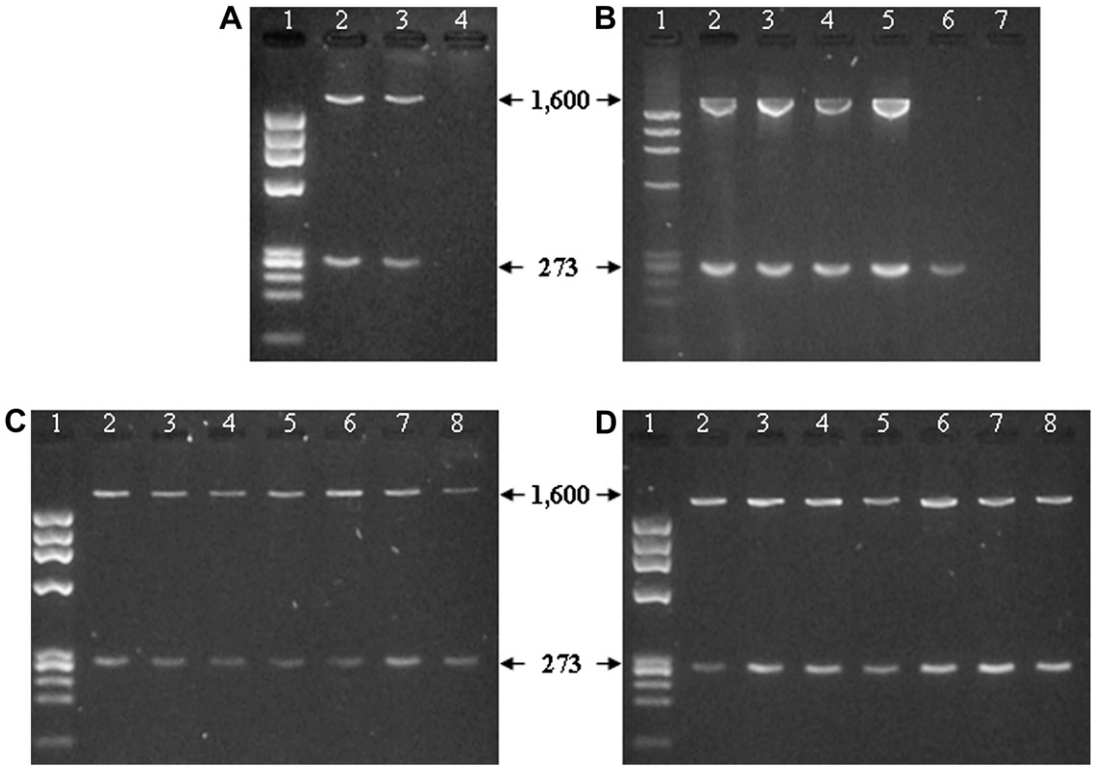

Fig. 1. PCR amplification of DNA from blood stains incubated at controlled relative humidity (RH) at room temperature (RT) or 35 °C. Panel A: RT and 100% RH (series II); lane 1, size standard (ΦX174 RF DNA *Hae*III-digested); lanes 2–4: 0, 2, 3 months. Panel B: 35 °C and 100% RH (series I); lane 1, size standard (ΦX174 RF DNA *Hae*III-digested); lanes 2–7: 0, 1, 2, 2½, 4, 6 months. Panel C: RT and 93% RH (series III); lane 1, size standard (ΦX174 RF DNA *Hae*III-digested); lanes 2–8: 0, 2, 3, 4, 6, 9, 12 months. Panel D: 35 °C and 93% RH (series III). Lane assignments as in C. Arrows indicate bands of amplified 1600 bp and 273 bp human genomic fragments.

method. It is noted that some extracts from later time points showed higher values. Whether this observation is caused by an increase in the extraction efficiency of older stains (e.g., because of decomposition of proteins and lipids trapping the nuclear DNA) or simply reflects a high variance between stains remains unresolved. However, overall the quantitative results confirmed that the stains contained sufficient DNA for adequate PCR amplification after 12 month at 50–93% RH at room temperature or 35 °C (>16,000 or >8200 copies of DNA/5 μl blood, respectively).

4. Discussion

Although DNA has a limited chemical stability[11] it has long been known that DNA in blood stains may remain relative stable for weeks, months, or even years at ambient temperature,[1–6] and samples (e.g., reference samples) that have been transferred to a matrix, e.g., FTA® paper, containing stabilizing agents are stable for at least 4 years.[18] Deposited biological matter is exposed to degradation by endogenic enzymes, microbial exoenzymes and spontaneous chemical reactions.[10,19] In the present work we have addressed the importance of air-humidity since the availability of water is necessary for both microbial activity and for un-catalysed hydrolytic reactions that are known to degrade DNA.[11,12] The influence of the humidity of the surrounding air on the stability of proteins was shown by Sensabaugh,[20] who observed an exponential increase in the inactivation rate of acid phosphatase in semen stains when RH was increased from 40% to 100%. It is therefore noteworthy that we found no indication that RH up to 93% had any significant effect on the stability of DNA in blood stains. The reason may be, that in contrast to the naked molecules of acid phosphatase, DNA in blood stains is most likely still entrapped in the nucleus and tightly bound to histones, which might offer some protection against degradation.[8] At the above mentioned conditions of humidity at room temperature, or 35 °C, DNA was amplifiable for at least seven months. This was supported by the observation that the number of template molecules did not drop significantly over time when RH $\leq$ 93%. This coincides with the absence of microbial growth on any of the stains incubated at RH $\leq$ 93%. It should be noted that even at humid locations around the World (Bergen, Brazzaville, Calcutta, Hong Kong, Kuala Lumpur and New Orleans) the average RH does not exceed 90%. Only Kuala Lumpur has a higher morning RH, but it drops considerable during the day, and the average 24-h RH is about 80%.[21] Therefore, even at locations with high humidity ambient conditions are adequate for long time storage of untreated stains. The present results are in agreement with previous observations obtained at ambient temperature.[1–6] In one study,[5] however, EDTA blood was used for the preparation of blood stains, and since EDTA is a biocide and inhibitor of metal-ion catalysed nucleases[22–25] the survival of DNA for 19 month is not directly comparable to the present results on blood and paper without any additives.

The reduced DNA stability at 100% RH confirms previous results for incubates at these conditions,[2] although in the present study amplifiable DNA still survived for months. The crucial point appears to be the onset of microbial growth, which happened at room temperature and at 35 °C. In the biologically less favourable temperature range (45–65 °C) microbial growth was not observed, but DNA was evidently less stable since the amplificability of the 1600 bp and 273 bp fragments dropped to approximately one month, and three months, respectively, at 55 °C and 65 °C. It is, however, noted that even at 45 °C, the 1600 bp fragment was amplifiable after eight months.

Given the high stability of DNA in dry stains at ambient temperature and humidity, it can be excluded that endogenic enzymes play any significant role in the degradation of DNA. Firstly,

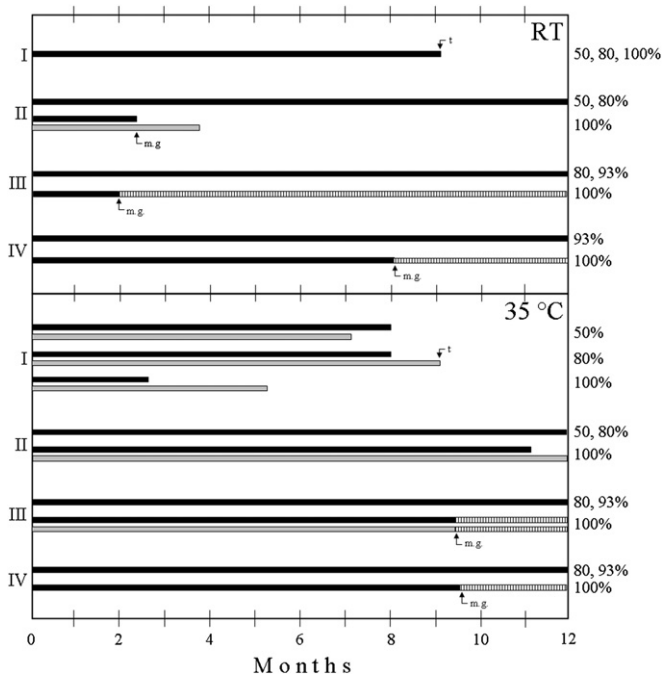

Fig. 2. Stability of DNA in blood stains at various conditions of relative humidity (RH) at room temperature (RT) or 35 °C. The diagram summarises PCR results for four independent series (I–IV) of stains comprising approximately 450 data points. The horizontal bars represent the observation of clearly visible bands after PCR amplification. Black bars, 1600 bp fragment; grey bars, 273 bp fragment. m.g., onset of microbial growth. t, termination of experiment. Hatched bars, microbial growth on some stains (but not all) and DNA extracted from those without growth. Series I and II were incubated at 50, 80 and 100% RH at either RT or 35 °C. Series III (exposed to the open air in a park) and IV were incubated at 80, 93 and 100% RH at either RT or 35 °C. The right *y*-axis shows RH values, identical results for a given series are grouped. When the results were the same for the long and the short fragment only results for the long fragment are shown.

the low quantity of water in "dry" stains presumably is too small to allow enzymatic reactions to occur, secondly, most endogenic enzymes become inactivated due to denaturation during the drying process. The present results indicate that degradation of DNA at lower temperatures is mainly exerted by the presence of microbial organisms. At higher temperatures (in the present work 45–65 °C), degradation of DNA is probably the result of spontaneous hydrolytic and oxidative reactions,[19] most notably depurination which results in subsequent breakage of the DNA strand.[11] Such non-enzymatic reactions are often more temperature sensitive than enzymatic processes; for example the rate of depurination increases 6–7 fold for each 10 °C the temperature is raised, as compared to ~2 fold for enzymatic reactions.[26–28] The result of these non-enzymatic reactions is the accumulation of nucleotide derivatives that affect the ability to PCR amplify the DNA, as well as an exponential increase in shorter and shorter DNA fragments.[11,29,30]

The present work shows that normal climatic conditions are not critical for the long time survival of DNA in untreated blood stains.

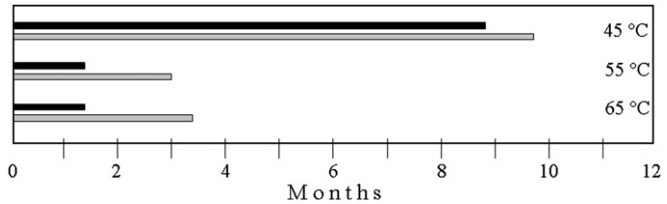

Fig. 3. Stability of DNA in blood stains at 45–65 °C and 100% relative humidity. The horizontal bars represent the observation of clearly visible bands after PCR amplification. Black bars, 1600 bp fragment; grey bars, 273 bp fragment.

This information may be useful for laboratories who wish to store reference blood samples in a simple way, while saving the costs of commercial substrates that are treated with DNA stabilizing agents. The amplicon size of standard forensic STR's are <500 bp,[31] and <300 bp for STRs tailored for degraded DNA (e.g., Minifiler®, Applied Biosystems, Foster City, CA), and for SNP-based systems the amplicon size is <150 bp.[6] We believe that the present findings, using a 1600 bp fragment that is much larger than any STR amplicon, and a 245 bp fragment larger than any SNP amplicon, provide an adequate assessment of the forensic applicability of DNA after long time storage of blood stains.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jflm.2010.08.001

Conflict of interest

We hereby declare that there are no conflicts of interests regarding this manuscript.

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None

Ethical approval

Not necessary

References

1. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA fingerprints. *Nature* 1985;**318**:577–9.
2. Prinz M, Staak M, Berghaus G. DNA extraction from bloodstains in respect to age and stained substrate. *Acta Med Leg Soc, Liege* 1989;**39**:213–20.
3. Cassol S, Salas T, Gill MJ, Montpetit M, Rudnik J, Sy CT, et al. Stability of dried blood spot specimens for detection of human-immunodeficiency-virus DNA by polymerase chain-reaction. *J Clin Microbiol* 1992;**30**:3039–42.
4. Kobilinsky L. Recovery and stability of DNA in samples of forensic science significance. *Forensic Sci Rev* 1992;**4**:67–86.
5. Kline MC, Duewer DL, Redman JW, Butler JM, Boyer DA. Polymerase chain reaction amplification of DNA from aged blood stains: quantitative evaluation of the "suitability for purpose" of four filter papers as archival media. *Anal Chem* 2002;**74**:1863–9.
6. Dixon LA, Murray CM, Archer EJ, Dobbins AE, Koumi P, Gill P. Validation of a 21-locus autosomal SNP multiplex for forensic identification purposes. *Forensic Sci Int* 2005;**154**:62–77.
7. McNally L, Shaler RC, Baird M, Balazs I, De Forest P, Kobilinsky L. Evaluation of deoxyribonucleic acid (DNA) isolated from human bloodstains exposed to ultraviolet light, heat, humidity, and soil contamination. *J Forensic Sci* 1989;**34**:1059–69.
8. Hall A, Ballantyne J. Characterization of UVC-induced DNA damage in bloodstains: forensic implications. *Anal Bioanal Chem* 2004;**380**:72–83.
9. Schwartz TR, Schwartz EA, Mieszerski L, McNally L, Kobilinsky L. Characterization of deoxyribonucleic acid (DNA) obtained from teeth subjected to various environmental conditions. *J Forensic Sci* 1991;**36**:979–90.
10. Eglinton G, Logan GA. Molecular preservation. *Phil Trans R Soc Lond B* 1991;**333**:315–28.
11. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 1993;**362**:709–15.
12. Poinar HN, Stankiewicz BA. Protein preservation and DNA retrieval from ancient tissues. *Proc Natl Acad Sci USA* 1999;**96**:8426–31.
13. Weast RC. *CRC handbook of chemistry and physics*. Boca Raton: CRC Press Inc;; 1989.
14. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science* 2001;**291**:1304–51.
15. Cheng CK, Chan J, Cembrowski GS, van Assendelft OW. Complete blood count reference interval diagrams derived from NHANES III: stratification by age, sex, and race. *Lab Hematol* 2004;**10**:42–53.
16. Perdigon HB, Calacal GC, Co Seng KL, Halos SC, De Ungria MCA. Evaluation and in-house validation of five DNA extraction methods for PCR-based STR analysis of bloodstained denims. *Sci Diliman* 2004;**16**:37–48.
17. Prośniak A, Gloc E, Berent J, Babol-Pokora K, Jacewicz R, Szram S. Estimating the efficiency of DNA isolation methods in semen, blood and saliva stains using the quantiblot system. *Arch Med Sadowej Kryminol* 2006;**56**:19–23.
18. Smith LM, Burgoyne LA. Collecting, archiving and processing DNA from wildlife samples using FTA® databasing paper. *BMC Ecol* 2004;**4**:4.
19. Gates KS. An overview of chemical processes that damage cellular DNA: spontaneous hydrolysis, alkylation, and reactions with radicals. *Chem Res Toxicol* 2009;**22**:1747–60.

20. Sensabaugh GF. The utilization of polymorphic enzymes in forensic science. *Isozymes Curr Top Biol Med Res* 1983;**11**:137–54.
21. BBC weather at, http://www.bbc.co.uk/weather/world/city_guides/.
22. Brul S, Stratford M, van der Vaart JM, Dielbandhoesing SK, Steels H, Klis FM, et al. The antifungal action of 1,10-o-phenanthroline and EDTA is mediated through zinc chelation and involves cell wall construction. *Food Technol Biotechnol* 1997;**35**:267–74.
23. Morrissey JV. Deoxyribonuclease II (DNase II), a zinc enzyme, and its inhibition by EDTA and 2-mercaptoethanol. *Am Chem Soc* 2005;**229**:U477.
24. Liaqat I, Bachmann RT, Sabri AN, Edyvean RG, Biggs CA. Investigating the effect of patulin, penicillic acid and EDTA on biofilm formation of isolates from dental unit water lines. *Appl Microbiol Biotechnol* 2008;**81**:349–58.
25. Al-Bakri AG, Othman G, Bustanji Y. The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin-EDTA combination and their effectiveness as antibiofilm agents. *J Appl Microbiol* 2009;**107**:280–6.
26. Lindahl T, Nyberg B. Rate of depurination of native deoxyribonucleic acid. *Biochemistry* 1972;**11**:3610–8.
27. Dissing J, Svensmark O. Human red cell acid phosphatase: quantitative evidence of a silent gene P^0, and a Danish population study. *Hum Hered* 1976;**26**:43–58.
28. Smith CI, Chamberlain AT, Riley MS, Stringer C, Collins MJ. The thermal history of human fossils and the likelihood of successful DNA amplification. *J Hum Evol* 2003;**45**:203–17.
29. Poinar HN. The genetic secrets some fossils hold. *Acc Chem Res* 2002;**35**:676–84.
30. Brotherton P, Endicott P, Sanchez JJ, Beaumont M, Barnett R, Austin J, et al. Novel high-resolution characterization of ancient DNA reveals C˙U-type base modification events as the sole cause of post mortem miscoding lesions. *Nucl Acids Res* 2007;**35**:5717–28.
31. Krenke BE, Tereba A, Anderson SJ, Buel E, Culhane S, Finis CJ, et al. Validation of a 16-locus fluorescent multiplex system. *J Forensic Sci* 2002;**47**:773–85.